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Phospholipid-induced structural changes to an erythroid β spectrin ankyrin-dependent lipid-binding site

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ABSTRACT

The region of β-spectrin that is responsible for interactions with ankyrin was shown to comprise an ankyrin-sensitive lipid-binding site. Structural studies indicate that it exhibits a mixed $3_{10}/\alpha$ helical conformation and is highly amphipathic. These features together with the distinctively conserved sequence of the lipid-binding site motivated us to explore the mechanism of its interactions with biological membranes. A series of singly and doubly spin-labeled erythroid β-spectrin-derived peptides was constructed, and the spin-label mobility and spin–spin distances were analyzed via electron paramagnetic resonance spectroscopy and two different calculation methods. The results indicate that in β-spectrin, the lipid-binding domain, which is part of the 14th segment, has the topology of typical triple-helical spectrin repeat. However, it undergoes significant changes when interacting with phospholipids or detergents. A mechanism for these interactions is proposed in this paper.

1. Introduction

The outstanding structural and mechanical properties of the red cell membrane are due to the presence of a regular protein network called the membrane skeleton on its cytoplasmic surface [1]. Spectrin, the major component of this skeleton, is a high molecular weight elongated protein formed by the head-to-head arrangement of two heterodimers composed of α (280-kDa) and β (247-kDa) subunits [2,3]. Each of the subunits predominantly forms a segmental triple-helical molecule [4], which also contains several other distinctive domains that determine spectrin's multifunctionality [5]. Detailed structural studies of the triple-helical segments, each of which is approximately 106 amino acid residues long, indicate that these are slightly distorted, left-handed coiled coils, with two parallel (A and C) and one antiparallel (B) helices [6–8]. The stability of the spectrin triple-helical repeats is assumed to be mostly dependent on inter-helical interactions [9,10]. Together with the relative arrangement and orientation of the repeats and the distinctive conformational properties of the helical linkers between the repeating units, these are thought to be key determinants of the structural and mechanical features of the spectrin tetramer [9–12]. Besides determining the spectrin structure, the repeats also serve as important docking sites for numerous structural and signaling proteins [13]. The most important example is ankyrin, which links the AE1 (anion-exchanger 1) protein with β-spectrin [14], yielding one of the major anchors between the membrane skeleton and the lipid bilayer. The ankyrin-binding site of β-spectrin includes highly conserved regions within the 14th and 15th repeats of the protein [15,16].

Spectrin–lipid interactions have been widely reported [17–22], and it is assumed that they exert an influence on the stability of biological membranes. One consequence of the multifunctionality of spectrin is that there are several lipid-binding activities connected with the occurrence of different binding sites [23,24]. The high affinity lipid-binding sites of spectrin seem to be confined to particular regions of the molecule [20–22]. One of these appeared to be the ankyrin-binding domain, as the binding of erythroid β-spectrin to phospholipid (phosphatidylethanolamine/phosphatidylcholine) vesicles or the monolayer was competitively inhibited by ankyrin [25,26]. Similar results were obtained for brain spectrin [19]. Detailed molecular analyses of a bacterially expressed full-length ankyrin-binding domain of erythroid spectrin and its truncated forms enabled us to locate an ankyrin-sensitive binding site for PE-rich lipids in a 38-residue N-terminal fragment of the domain, with the eight initial amino acid residues playing a key role in this activity [27]. Moreover, as the whole-length ankyrin-binding domain, unlike its truncated forms, induced changes in cell morphology and aggregation on the

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membrane skeleton during its transient expression in eukaryotic cells, the 38-residue fragment seems to be the major element of mechanisms regulating the membrane skeleton in vivo [28].

Most recently, we managed to solve the structure of the ankyrin-sensitive binding site for PE-rich lipids using the site-directed spin-labeling technique [29]. The analyzed fragment exhibits a helical conformation revealing a distinct 3_{10}-helix contribution at its N-terminus. The specific conformation is correlated with the highly amphipathic character of the whole structure of the lipid-binding region. Although our previous work yielded a lot of structural details about the described site, a continuation appeared to be needed to create an unequivocal picture of the lipid-binding activity of erythroid β-spectrin.

To address this issue, we prepared a series of singly and doubly spin-labeled mutants based on a peptide comprising the whole 14th segment framed with shorter fragments of adjacent spectrin repeats of the erythroid β subunit. The peptides were subjected to EPR (electron paramagnetic resonance) spectroscopy and subsequent spectral analyses: spin–spin distance calculations via the Fourier deconvolution method [30,31] and spin-label mobility reflected by scaled mobility [32]. Together with those previously described [29], our current results indicate that although the lipid-binding domain of β-spectrin is a part of the 14th segment which exhibits structural features typical for a triple-helical spectrin repeat, it undergoes considerable changes in its tertiary structure when interacting with phospholipids or detergents. The interactions with phospholipid bilayers are thought to be lateral, and it is during these that the induced exposition of the hydrophobic surfaces of the helices facilitates their binding to the core of biological membranes.

2. Materials and methods

2.1. Construction, isolation, spin-labeling and characterization of mutated peptides

The template for the introduced substitutions was the pRSET vector containing the gene for the erythrocyte β-spectrin-derived peptide (amino acid residues 1638–1845 according to the sequence gi:134798). Site-directed mutagenesis was performed using a QuikChange® kit (Stratagene, La Jolla, USA), yielding seven double-cysteine and nine single-cysteine variants, and one variant with cysteine and serine substitutions. The mutations were confirmed via DNA sequencing. The desired peptides were expressed in *E. coli* BL21(DE3) pLysS cells (Promega), and subsequently isolated as described elsewhere [27]. The peptides were subjected to SDS-PAGE (polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate) to check their purity and molecular weight.

Following purification, the mutated peptides underwent the spin-labeling procedure. The first step was incubation with dithiothreitol (10 mM) overnight at 4 °C and its subsequent removal using a Bio-Silect SEC 250-5 column (Bio-Rad, Hercules, USA) in an FPLC (fast protein liquid chromatography) system. A 20-fold excess of MTSL (3-metylthiosulfonyl-1-oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline; Toronto Research Chemicals, North York, Canada) [33] was used to selectively spin-label the mutated peptides. Thus, the cysteine residues were substituted by an R1 side chain. The samples were concentrated and the reaction was continued overnight at 4 °C in the presence of GuHCl (guanidine-hydrochloride), which appeared to improve the spin-labeling efficiency by exposing the buried amino acid residues to the spin label; this step did not affect the measured features of the peptides. Finally, unbound MTSL and GuHCl were removed by another run of the Bio-Silect column. Alternatively, desalting steps were performed using Vivaspin 500 10000MWCO (Sartorius, Germany) ultrafiltration spin columns.

The influence of mutation and spin-labeling on the structural properties of the peptides was verified by CD (circular dichroism) measurements performed on a Jasco 715 CD spectrometer. A temperature-controlled cell with a 0.1-cm path length was used at 10 °C, or in the range of 10 to 70 °C with 10 °C increments and the samples were in a 20 mM Tris–HCl/100 mM NaCl, pH 7.5 buffer. The percentage of helicity was calculated from the values of the amide $n\pi^*$ transition at 222 nm ($[\Theta]_{222}$), assuming that a value of $-36{,}000°\ cm^2\ dmol^{-1}$ represents 100% α-helical content.

Absorbance measurements at 280 nm or the Bradford method (Bio-Rad) were employed to determine the protein concentration. The concentration of free cysteine –SH groups after spin labeling was assayed with DTNB (5, 5′-dithiobis-(2-nitrobenzoic acid) — Ellman's reagent; P-L Biochemicals, Milwaukee, USA), and the spin-labeling efficacy was calculated as follows: 100% [(total cysteines)−(free cysteines)]/(total cysteines).

Monolayer experiments, performed using the Wilhelmy technique on a Nima tensiometer (Nima technology, Coventry, UK) with a Teflon trough with a 24-cm^2 surface area, were carried out as previously described [27].

2.2. Reconstitution of the spin-labeled peptides into lipid bilayers

The procedure of reconstitution was performed according to Mimms et al. [34]. Approx. 4% of octylglucoside nonionic detergent was added to PE/PC (3/2 mol/mol) (Lipid Products, UK) MLV (multilamelar vesicle) liposomes suspended in a solution of spin-labeled peptide. After a short incubation at room temperature, the detergent was removed using an FPLC system equipped with a Bio-Silect SEC 250-5 column. Fractions of the protein attached to liposomes were collected and concentrated. The amount of residual octylglucoside was determined via phenolic assay [35].

2.3. EPR measurements

EPR spectra were obtained on a Bruker ESP 300E 9 GHz spectrometer. For spin–spin distance measurements, samples of approximately 200–350 μM in a 20 mM Tris–HCl/100 mM NaCl, pH 7.5 buffer were frozen in liquid nitrogen in a Dewar flask. EPR spectra were acquired using a 1 G field modulation amplitude at 100 kHz and a 0.2–2 mW incident microwave power. A magnetic sweep width of 200 G was used. Each time, a total of nine scans were accumulated.

For side-chain mobility experiments, the following modifications of the above were applied: spectra were acquired at room temperature, the incident microwave power reached 10 mW and the sweep width was 100 G. All the measurements were performed at least in triplicate.

2.4. Spin-label mobility determination

The EPR line shape of the spin label is sensitive to the local environment. We used the inverse of the width of the central resonance line (ΔH_0^{-1}) in a normalized form to give a scaled mobility (M_S). The latter is defined as $M_S=(\Delta H_0^{-1}-\Delta H_{0i}^{-1})/(\Delta H_{0m}^{-1}-\Delta H_{0i}^{-1})$, where ΔH_0^{-1} is the width of the central resonance line of R1 at the analyzed site, ΔH_{0i}^{-1} is the corresponding width for the most immobilized side chain, and ΔH_{0m}^{-1} is the corresponding width for the most mobile R1 in the peptides. Each spin-labeled peptide was represented by the average value from two or three data sets.

2.5. Spin-label separation calculations

Two methods based on Fourier deconvolution of EPR spectra were employed for the spin-label separation calculations. The main idea of both is to compare the spectra for the doubly spin-labeled proteins with the corresponding singly labeled samples with non-interacting spins [36]. The EPR "Spectroscopic ruler" method [30] treats the EPR spectrum of the two interacting spins in doubly labeled peptides as a convolution of the spectrum of non-interacting spins in the singly labeled peptides with a dipolar broadening function.

Assuming that the broadening function can be used to estimate the average splitting values (<2*B*>) for doubly labeled peptides, and that the coupling between the two spins is presumed to be axially symmetric, the mean spin–spin distance (<*r*>) was determined according to the equation: $\langle r\rangle = [(0.75)(3/2g_e\beta/\langle 2B\rangle]^{1/3}$, where g_e is the isotropic *g* value for electrons and β is the electron Bohr magneton. The cutoff frequency for the determination of the Gaussian fit broadening function in the Fourier space was at points 20–40; thus, the reported average distances were accompanied by error ranges observed for several cutoff frequencies applied to several data sets. The method can separate the dipolar spectrum for the interacting nitroxides from the spectral contribution from monoradical species, as the latter contributes to the *y*-axis offset in the Fourier space. The numerical data was analyzed using Origin 4.0 (Microcal, Northampton, USA) and Excell (Microsoft Corporation).

An alternative approach involves fitting the simulated dipolar broadened spectrum to the experimental one, where the first is convoluted [31]. Thereby, the average distance and the width of the Gaussian distance distribution between the attached spin labels was calculated from the fitted line width function using the *Dipfit* software developed by the authors of the method. Using the software, it is also possible to define the amount of monoradical impurities which contribute to the recorded spectra.

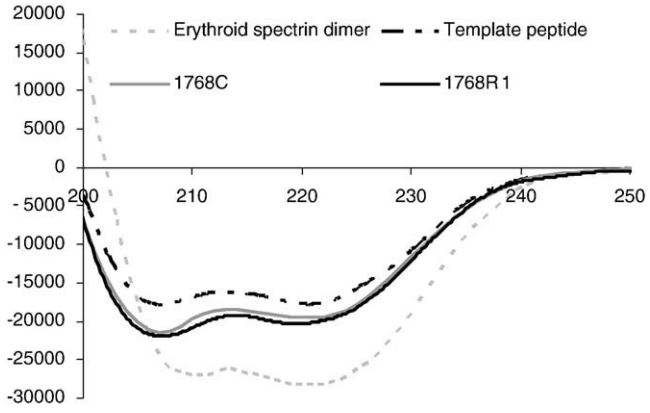

Fig. 1. Examples of the CD spectra obtained for erythroid spectrin, a template β-spectrin peptide comprising residues 1638–1845, and one of the mutated peptides before and after spin labeling; the latter is representative for all the mutants prepared. It appeared that neither introduced mutations nor spin labeling perturb the structure of the protein. All the spectra were measured at 20 °C. The *X*-axis is the wavelength in [nm] and the *Y*-axis is the molar ellipticity [degrees cm^2 $dmol^{-1}$]. The average helical contents of the mutated peptides were 54.3±7%, which was comparable with those for the non-mutated peptide.

3. Results

3.1. Preparation and characterization of spin-labeled peptides

We focused on the most recently described ankyrin-sensitive lipid-binding domain of β-spectrin [27] in the context of the whole 14th repeat domain. A series of ten single-cysteine mutants together with seven double-cysteine mutants within a peptide containing amino acid residues 1638–1845 of the β subunit of human erythrocyte spectrin were prepared. The mutants were subsequently spin labeled with MTSL, yielding the following peptides: 1756R1, 1761R1, 1768R1, 1769R1, 1770R1, 1771R1, 1779R1, 1784R1, 1797R1, 1778S/1779R1, 1699R1/1768R1, 1710R1/1779R1, 1750R1/1771R1, 1754R1/1761R1, 1754R1/1768R1, 1754R1/1784R1 and 1771R1/1779R1. Thus, constructing singly spin-labeled peptides, we focused on a stretch of the sequence 1756 RLIDAGHSEAATIAEWKDGLNEMWADLLELIDTRMQLLAASY 1797 comprising the binding site for PE-rich lipids [27], the detailed secondary structure of which was recently determined in our lab [29]. Analyzing the spectral properties of the spin labels at the targeted sites should enable us to determine the topographical characteristics of the structural motif. Moreover, the sites for double spin labeling were designed in order to monitor the distances between the ankyrin-sensitive lipid-binding domain and the two other helices of the 14th repeat domain of erythroid β spectrin.

All the peptides expressed and isolated from bacteria were electrophoretically pure. The results of the CD experiments (Fig. 1) indicate that all the mutated peptides were well folded with nearly identical spectral shapes to the template β-spectrin peptide. Neither cysteine replacement nor spin labeling affected their general conformation, which was estimated to be helical in the range of approximately 50 to 60%. Moreover, sigmoidal-shaped temperature-dependent denaturation profiles were recorded. Monolayer experiments applied to the randomly selected peptides revealed that none of them lost their affinities to PE/PC mixtures of lipids and ankyrin. Assays for free cysteines combined with protein concentration analyses indicated that the spin-labeling efficiencies for the double-labeled peptides were more than 80%.

3.2. Mobility of the spin labels

In our previous study, we managed to determine the structure of the ankyrin-sensitive binding site for PE-rich lipids by spin–spin distance measurements [29]. This fragment possesses a helical conformation with a distinct 3_{10}-helix contribution at its N-terminus, and is entirely composed of helix C of the 14th repeat unit of erythroid β spectrin. As the high amphipathicity of the helix is evident, we tried to determine which of the amino acid residues of the region are exposed and which are involved in interhelical contacts. Therefore, the mobility of spin labels attached to different positions within the ankyrin-sensitive lipid-binding site were analyzed. The room temperature EPR spectra of singly labeled peptides exhibit various line shapes depending on the position of the spin label in the sequence. Strong interactions of the nitroxide side-chain R1 with neighboring atoms, as indicated by a broadened line and increased apparent hyperfine splitting, are evident for 1768R1, 1769R1, 1771R1, 1779R1, 1797R1 (Fig. 2 C, D, F, G and J). These positions seem to be those engaged in the strong interhelical contacts of 14th triple-helical repeat, as the side chains of neighboring positions in the sequence exert only a weak reciprocal effect. This is evident when comparing the R1 side-chain spectra of 1779R1 and 1778S/1779R1 (Fig. 2 G and H), the latter with the M residue substituted to S in one position upstream to the R1 side chain. On the other hand, the spin labels attached to 1770R1 and 1784R1 display a high degree of mobility, which results in the small apparent hyperfine splitting and line width of the EPR spectrum (Fig. 2 E and I). Positions 1770 and 1784 were originally occupied by negatively charged residues and are believed to be exposed to the exterior of the protein. The spectra of 1756R1 and 1761R1 represent side chains of intermediate mobility (Fig. 2 A and B). The observed differences in the EPR line shape were considerably reduced in the presence of 6 M urea, with an overall decrease in line broadening in all cases (see Fig. 2). Thus, the observations undoubtedly reflected the structural features of the native peptides. Moreover, plotting the resulting scaled mobility values as a function of residue number nearly perfectly matches the periodicity of the side chain mobility predicted according to the previously described structural model of the ankyrin-dependent lipid-binding site of erythroid β-spectrin (Fig. 3 A) [29].

The most intriguing effects were the observed dramatic changes of the EPR spectral line shape induced by the addition of nonionic detergent (octyl glucoside) or the reconstitution of spin-labeled peptides to liposomes (see Fig. 2). There was a distinct gain of mobility, particularly for the spin-label occupied “hydrophobic” positions of the amphipathic helix and to a lesser extent for the spin labels located in

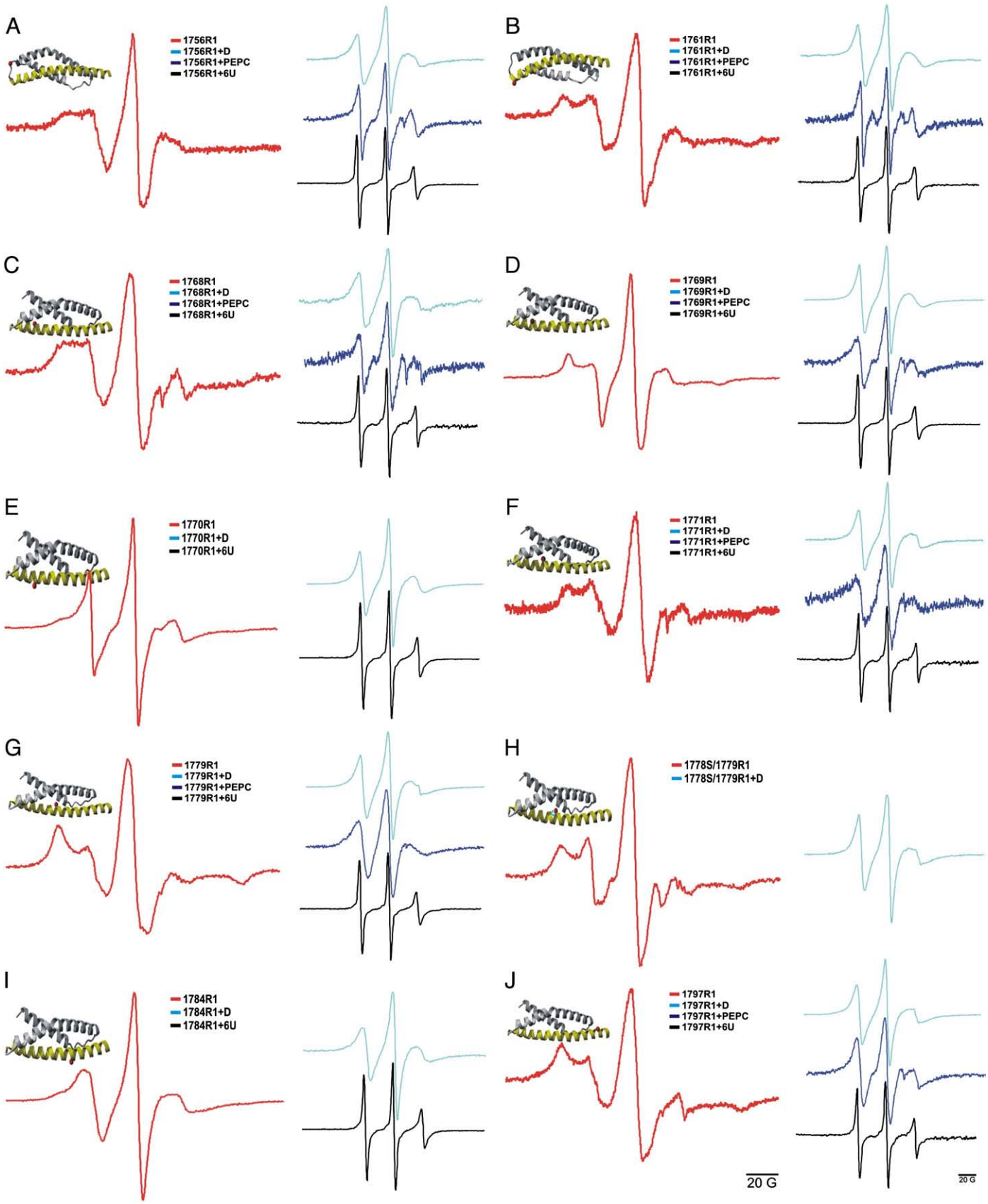

Fig. 2. Room-temperature EPR spectra of MTSL singly labeled mutants (red) originating from β-spectrin-derived peptide. The spectra of 6 M urea-denatured peptides (+6U) are in black, those recorded in the presence of 2% octyl glucoside (+D) are in light blue, and those for peptides reconstituted in PE/PC liposomes (+PEPC) are in dark blue. The *X*-axis is the magnetic field [Gauss]. Each set of spectra is accompanied by the rough 3D model of the corresponding peptide with the location of spin label marked in red.

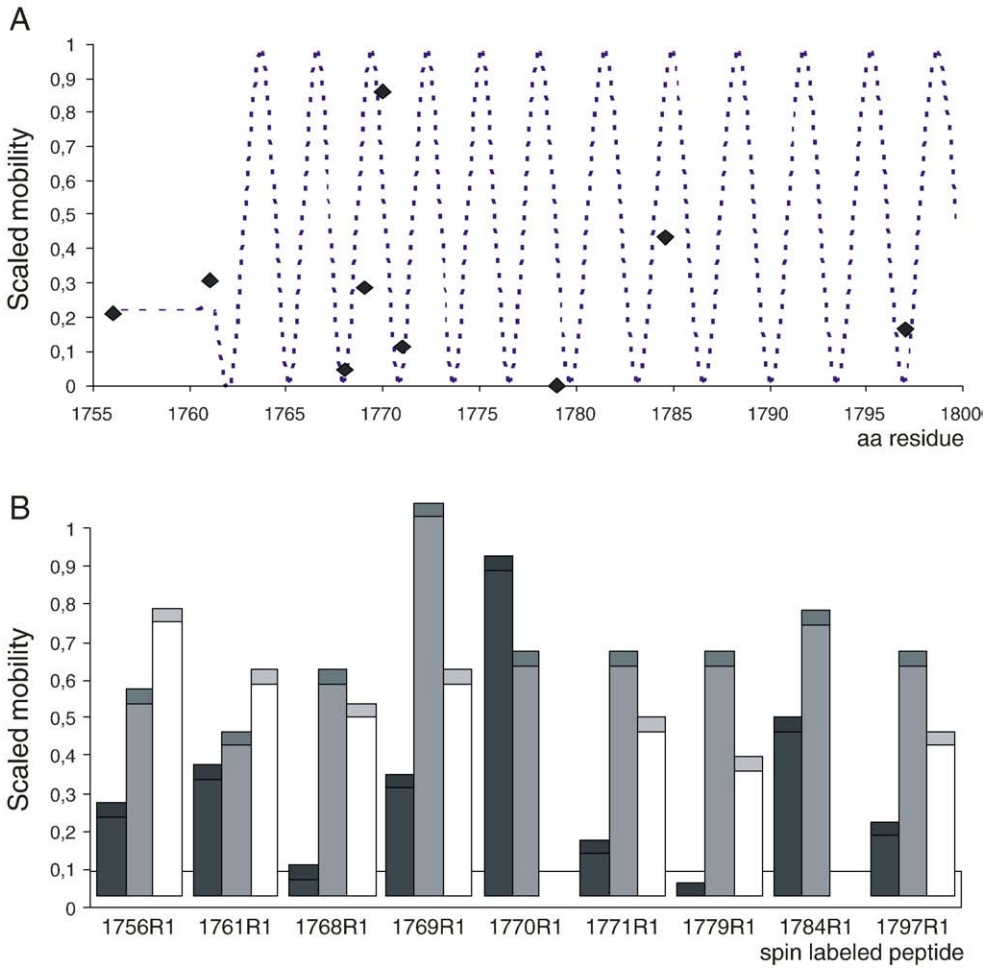

Fig. 3. Spin-label mobility determination. (A) The scaled mobility of the R1 side chains plotted as a function of the residue number of amino acids substituted by spin labels; the dotted line represents the theoretically predicted fluctuations according to the model of the ankyrin-dependent lipid-binding domain of β-spectrin [27], where the α-helix comprises residues 1774–1801, and the 3_{10}-helix consists of residues 1760–1773 and a loop is upstream from the latter. (B) The mobility changes induced by 2% octyl glucoside (light grey) or liposomes PE/PC (3:2 mol/mol; white) when compared with non-treated spin-labeled peptides (grey).

the preceding loop (Fig. 3 B). Thus, the binding of amphipathic ligands is most probably coupled with changes in the tertiary structure of the 14th repeat domain of erythroid β-spectrin.

3.3. Spin–spin distance analysis

To further investigate the structural changes, we attempted to follow the changes in the interhelical contacts by spin–spin interaction analysis. Spin labels were introduced into the peptides to measure the distances between the helices of the 14th repeat domain of β-spectrin. The spin–spin interaction is manifested by EPR spectral broadening, which enables the measurement of distances [30,31]. However, the line broadening caused solely by the spin–spin interaction is better represented in the EPR spectra taken under rigid solution conditions. In the comparison of the spectra of doubly labeled peptides with those of singly labeled peptides recorded at liquid nitrogen temperature, various extents of line broadening due to spin–spin interactions were observed (Fig. 4). Moreover, pronounced spectral broadening changes could be observed in the presence of 2% octyl glucoside or after the reconstitution of peptides in PE/PC liposomes. Together with the quantitative examination of the structure by the distance determination (Fig. 5), these observations unambiguously demonstrate that the 14th β-spectrin repeat possesses a typical, triple-helical structure where amino acid residues occupying distant positions in the sequence are brought together in the native structure. However, the structure is not static and is strongly influenced by the amphipathic molecules of nonionic detergents or phospholipids: in the case of 1699R1/1768R1 and 1710R1/1779R1, both bearing one spin label on helix A and one on helix C, the respective distances of approximately 12 Å and 18 Å, become extended beyond the upper limit of the used method (>25 Å). Under the same conditions, the R1 side chains located on helix B move away from the spin-labeled helix C, albeit to a lesser extent (peptides: 1750R1/1771R1, 1754R1/1761R1 and 1754R1/1768R1). The smaller amplitude of movement in the latter case is probably due to the proximity of a loop which connects the two helices. On the other hand, two spin labels located on opposite ends of the repeat (1754R1/1784R1) did not interact with each other, but when R1 side chains are eight sequence positions away within helix C (1771R1/1779R1), their weak interactions are insensitive to the detergent and the liposomes. This is consistent with the previously proposed structural model [29] and further confirms that binding amphipathic ligands does not influence the secondary structure of the ankyrin-dependent lipid-binding domain, but it is coupled with changes in the tertiary structure of the 14th repeat domain of erythroid β-spectrin. The mechanism of the latter is based on unwrapping (at least partial) of the triple-helical motif, which may lead to the subsequent exposition of the buried region of the structure (Fig. 6). The latter, particularly

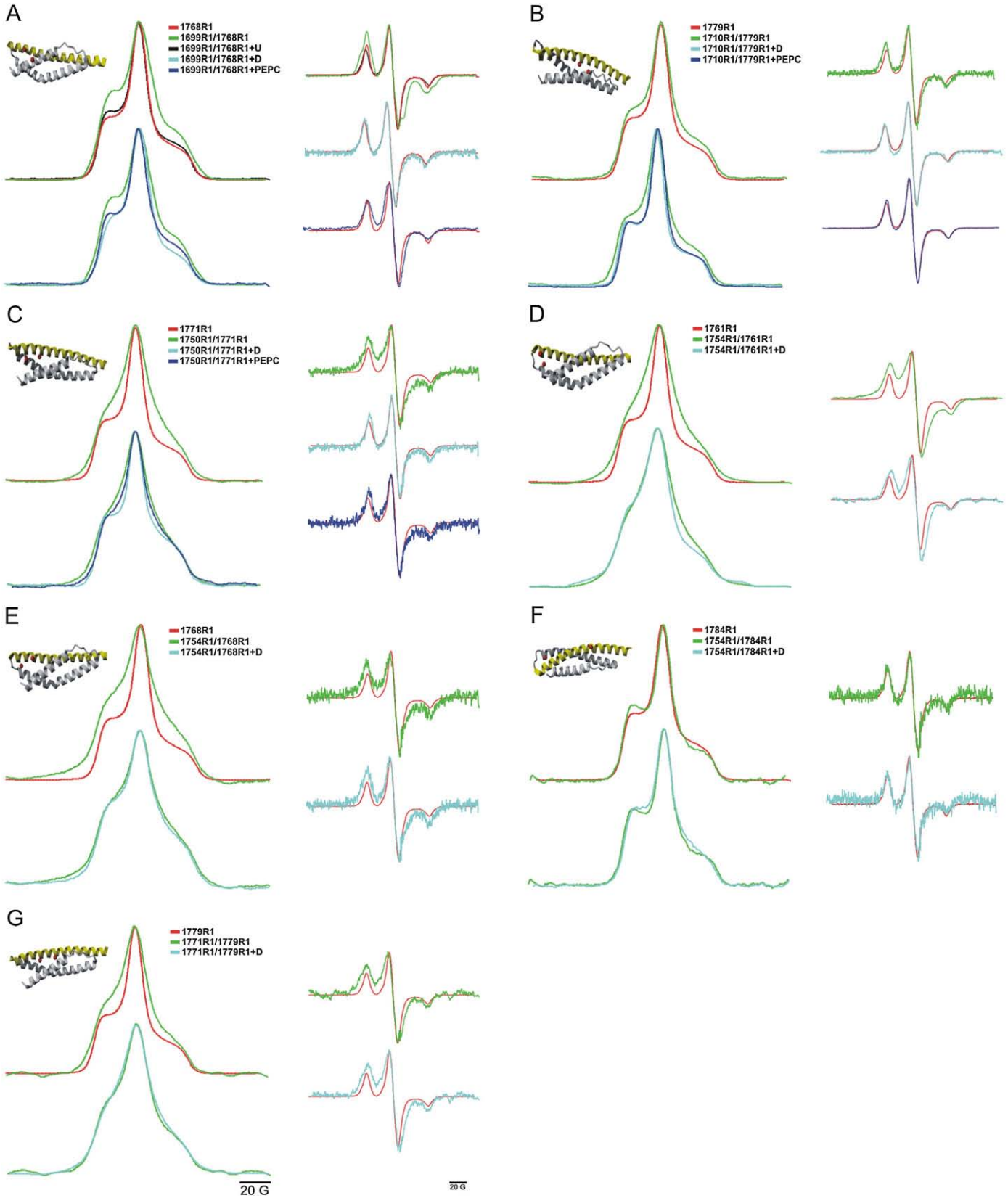

Fig. 4. A comparison of the low-temperature EPR spectra of MTSL doubly labeled mutants (green) with the spectra of singly labeled mutants (red), all originating from β-spectrin-derived peptide. One spectrum of 6 M urea-denatured peptides (+6U) is in black, those recorded in the presence of 2% octyl glucoside (+D) are in light blue, and those for peptides reconstituted in PE/PC liposomes (+PEPC) are in dark blue. The *X*-axis is the magnetic field [Gauss]. Each set of spectra is accompanied by the rough 3D model of the corresponding peptide with the locations of spin labels marked in red.

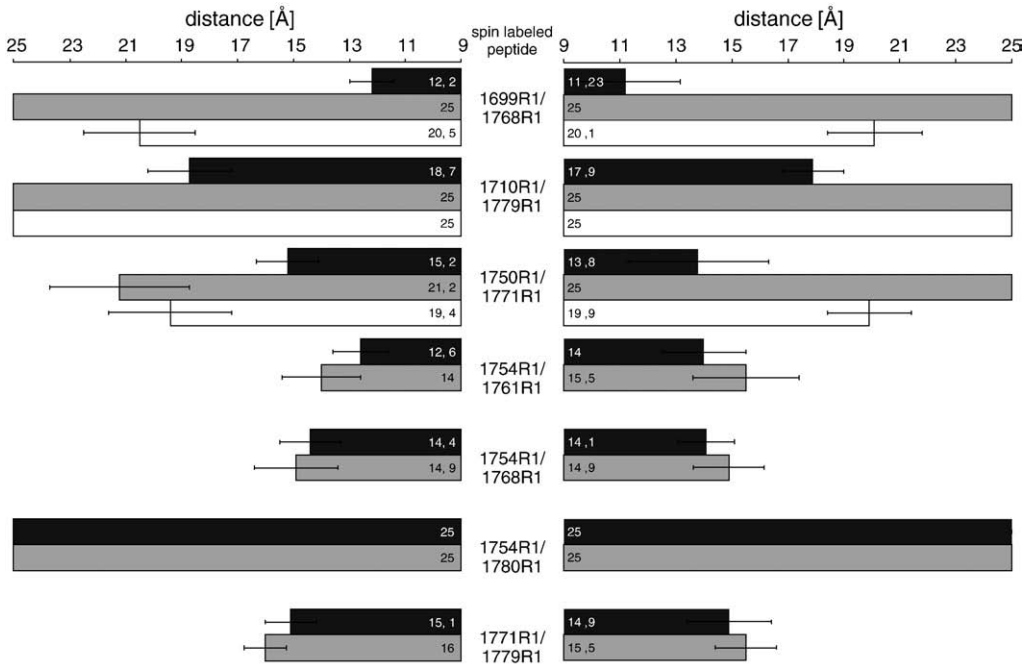

Fig. 5. Spin–spin distance analysis. (Left) The values of the average spin–spin distance for doubly labeled β-spectrin-derived peptides calculated via the "spectroscopic ruler" method [28]; the bar on the chart indicates experimental error. (Right) The values of the interspin distance for the same doubly labeled peptides calculated using *Dipfit* software [29]; the bar on the chart indicates the half width of the Gaussian function describing the calculated distance distribution between the attached spin labels. The distances calculated for the pure spin-labeled peptides are in dark grey; those in the presence of 2% octyl glucoside are in light grey, and those for peptides reconstituted in PE/PC liposomes are in white. Distances indicated as 25 Å exceeded the limit of the applied method.

the hydrophobic surface of the extremely amphipathic helix C then becomes free to interact with the core of biological membranes.

4. Discussion

Recently, we managed to localize an ankyrin-sensitive lipid-binding site of an ankyrin-binding domain in a typical triple-helical spectrin repeat rod region of erythrocyte β-spectrin (helix C of the segment 14) [29]. As this fragment is formed by a characteristic $3_{10}/\alpha$-helical conformation and its amino acid sequence exhibits a high degree of conservativity [16], it appeared encouraging to follow the mechanism of its interaction with lipids. To achieve this, we applied SDSL (site-directed spin labeling) combined with EPR spectroscopy to study key aspects of the tertiary structure of the domain and its changes during lipid binding with high precision. Its widespread applications have established SDSL as a well-developed structural biology technique [36] that can be used not only to obtain the structure of a protein but also to elucidate the mechanisms of its biological activity [37–39], which is often unattainable using other biophysical techniques. It should be emphasized that substitution with an R1 side chain exerts a negligible influence on the biological activity and stability of the analyzed protein, since its properties are described as similar to A, L and M residues [40]. Spin-label mobility reflected by scaled mobility [32] has the advantage of reflecting changes in both the order parameter and effective correlation time. It is simple to measure and reliable even in noisy EPR spectra with compromised baseline stability [41]. Dynamic sequences identified by site-directed spin labeling helped us to confirm the topography of the secondary structure of the analyzed lipid-binding domain. The observed changes in the line shapes of singly labeled mutants of the lipid-binding domain of erythroid β-spectrin upon binding to liposomes are convergent with the presented results from the detergent-treated spin-labeled mutants. Thus, it is unlikely that these are a consequence of the reduction in global tumbling due to protein–liposome interaction, but reflect changes in tertiary interactions. Spin–spin distance calculations via the Fourier deconvolution method have proven to be effective in the range of 7–25 Å with a standard deviation of 0.9 Å for the entire range [30,42,43]. However, to enrich the obtained data and get a more exact view of the observed structural changes, we also applied another widely used approach developed by the Steinhoff group [31,39,44]. This allowed us to obtain detailed and trustworthy data concerning the measured distances. Some authors reported that freezing the samples could affect the proteins' conformations [45], but we previously proved that this is not the case in our studies [29]. As spectrins are well known laterally

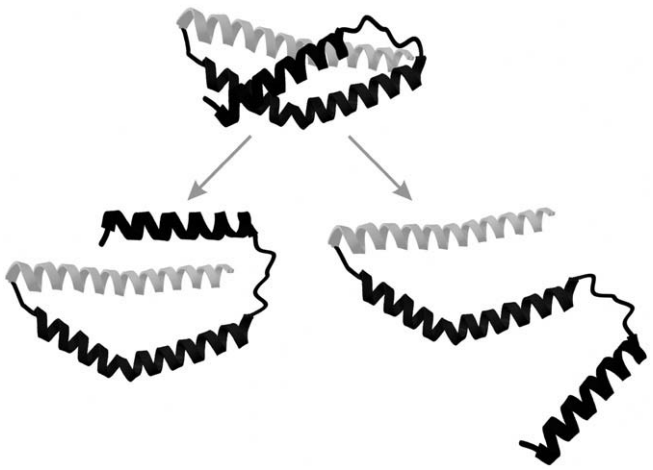

Fig. 6. A ribbon representation of the structure of the 14th segment of erythroid β-spectrin, with helix C in grey, showing the two possible pathways for partial unfolding events accompanying the interactions of the ankyrin-dependent lipid-binding domain with detergents or phospholipids. The accurate extents of interhelical distances increases are not measurable using the applied techniques.

dimerizing proteins, a possible aggregation of the examined peptides may occur. However, no EPR spectral broadenings were observed for singly labeled peptides, and the line shapes did not change upon the denaturation of these peptides, which gives evidence against the aggregation of the peptides used in our study. Moreover, the performed size exclusion chromatography of the denatured and non-denatured peptides resulted in nearly identical retention times in both cases.

Here, we estimated the general three-dimensional structure of the 14th repeat domain of erythroid β-spectrin comprising an ankyrin-dependent lipid-binding domain, emphasizing the role of the hydrophobic amino acid residues of the domain in protein stabilization and protein–lipid interaction. It appeared that the analyzed domain is a part of a regular triple-helical segment of spectrin, although phospholipids and detergents induce significant changes in the characteristic arrangement of the three helices. The proposed mechanism seems to be reasonable, as it is well documented that the same processes are responsible for the outstanding elasticity of spectrin [9,46]. It is also worth mentioning, that the 14th repeat domain of erythroid β-spectrin is ranked as an element of medium stability, highly susceptible to unfolding in physiological conditions [10]. Interhelical interactions are fundamental for spectrin repeat stability, and the majority of these are van der Waals contacts, where highly conserved W residues play a central role, with a small amount of electrostatic and hydrogen bond interactions [8,9]. This is consistent with our findings that the hydrophobic residues of the amphipathic helix of the ankyrin-dependent lipid-binding domain are the core elements of the interhelical contact area, while the charged side chains are on the exterior surface of the protein and are most probably involved in the docking of other proteins or ligands. However, the changes in the arrangement during lipid interactions lead to the exposure of hydrophobic core, allowing the domain to interact directly with biological membranes. Concerning the highly amphipathic character of the helix, its interactions with phospholipid bilayers are thought to be lateral, as was previously suggested in the context of some analogy to melitin [27]. Most probably, the inhibitory effect of ankyrin on the described interactions is due to a sterical hindrance of the protein which blocks the movements of the helices. However, the exact mechanism of binding ankyrin to spectrin still remains to be elucidated.

As far as the physiological significance of the double activity of the examined region of erythroid β-spectrin is concerned, we have proposed that PE-rich domains would serve as a membrane attachment site for spectrin in situations in which ankyrin is either deficient or its affinity for spectrin is reduced [23,47]. In these situations, the ankyrin-dependent PE-rich lipid-binding activity of spectrin would serve as anchor ensuring preservation of the mechanical properties in the membrane-skeletal lattice.

In conclusion, our results indicate that the lipid-binding domain of β-spectrin is a part of 14th segment, which exhibits structural features typical for a triple-helical spectrin repeat. The hydrophobic side chains of the amphipathic helical domain are involved in core formation, and the charged side chains are exposed to the exterior medium. The described structure undergoes significant changes at the level of its tertiary structure when interacting with phospholipids or detergents. Such interactions lead to the exposure of hydrophobic core of the segment, thus enabling the hydrophobic surfaces of the helices to laterally penetrate the interior of phospholipid membranes.

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